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METHODS FOR PREDICTING SURVIVAL RATE IN PATIENTS HAVING MULTIPLE SCLEROSIS

[0001] This invention claims priority to co-pending U.S. Provisional Application Serial No. 60/399,157 filed July 30, 2002.

Field of the Invention

[0002] This invention relates to genotyping and particularly to the detection of a genetic variant for predicting survival rate or time in patients having multiple sclerosis.

Background of the Invention

[0003] Multiple Sclerosis (MS) is a chronic demyelinating disorder pathologically characterized by an infiltration of monocytes and T-lymphocytes into the brain parenchyma, destruction of oligodendrocytes and the loss of myelin. The role of chemokines and chemokine receptors is particularly important in MS, in which myelin-destructive inflammation occurs inside the blood-brain barrier and is related to influx of peripheral pro-inflammatory T cells into the CNS. Chemokines play a significant role in the migration of monocytes and T cells and also have been implicated in the onset or progression of MS and experimental autoimmune encephalomyelitis (EAE) (Ransohoff, R.M., et al. 1999; Karpus, W.J. et al., 1995).

[0004] Causes of death in MS cases have not been well studied, although the loss of myelin and the subsequent

loss of ability to maintain adequate heat reaction and response to infections is likely to play a role. In a study of 6,068 MS cases from the Danish Multiple Sclerosis Registry, MS was noted on the death certificate as the underlying cause of death in 55.4% of cases (Koch-Henriksen N, et al., 1998), but the mechanism by which MS contributes to 'premature' death has not been explicated. However, young age at onset and an initial remitting clinical course have both been shown to be significantly associated with longer survival (Midgard R, et al., 1995). To date, studies on survival of MS patients in relation to genotypes have not been specifically addressed.

[0005] Chemokine receptor 5 (CCR5), a seven transmembrane-spanning G-protein coupled receptor, is a specific binding site for the CC-chemokines and is expressed on T-helper-1 (TH1) but not on T-helper-2 (TH2) subsets of lymphocytes. Several studies have ascribed to *CCR5* surface expression levels an important role in HIV-1 entry and pathogenesis (Huang Y, et al., 1996), and a *CCR5*Δ32 mutation (homozygous deletion) almost invariably protects from HIV-1 infection. (Biti R., et al., 1997; O'Brien TR et al., 1997). The heterozygotes demonstrate a delay in progression of the disease (deRoda Husman A-M, et al., 1997; Mummidi S., et al., 1998; Daar ES, et al., 1999), lower viral loads and higher CD4+ counts (Cone L.A., et al., 2000). Aberrant production of chemokines has been described in humans and experimental CNS demyelinating lesions (Balashov KE, et al., 1999; Sorensen TL, et al., 1999). Beta-chemokine receptors were examined in post-mortem MS CNS tissue by immuno-histochemistry, and an elevated expression of *CCR2, CCR3*

and *CCR5* was noted (Simpson J., et al., 2000; Zhang GZ, et al., 2000; Strunk T, et al. 2000). This over-expression could be due to a viral infection such as HHV6 that has been proposed to be associated with MS (Challoner PB, et al., 1995; Sanders VJ, et al., 1996; Knox KK, et al., 2000).

[0006] The ligands for *CCR5* include RANTES (regulated-upon-activation, normal T expressed and secreted), macrophage inflammatory protein-1 alpha (MIP-1 alpha) and MIP-1 beta. These ligands belong to the group of CC or beta chemokines and are found principally on TH1 helper cells. (Bonecchi R, et al., 1998). Earlier studies in HIV infected individuals have shown that T-cells from *CCR5*Δ32 heterozygotes express 5- to 10-fold less *CCR5* than wild types after stimulation and, in addition, CD4+ T-cell clones from two homozygous persons for the *CCR5*Δ32 mutation produced approximately 20-fold more RANTES than wild type clones (Paxton WA, et al. 2001). Analysis of cytokine and cytokine receptor gene expression in MS samples showed predominantly increased levels of several Th1 molecules (TGF-ss, RANTES, and macrophage-inflammatory protein (MIP)-1alpha), although some Th2 genes (*IL-3, IL-5, and IL-6/IL-6R*) were found to be up-regulated as well (Baranzini SE, et al., 2000).

[0007] Genetic studies reveal MS to be polygenic, due to multiple gene associations (Ebers GC, et al., 1994; Oksenberg JR, et al., 1996; Sawcer, S, et al., 1996). Reports of epidemiologic studies of MS (Poser CM, 1994) concluded that the disease was common in persons of Scandinavian descent: an ethnic group that exhibits a high prevalence of *CCR5*Δ32 mutation. Among the European white population there is a north to south gradient of

prevalence of the *CCR5*Δ32 mutant allele, with the allelic frequencies highest in Scandinavia (16%) and lowest in Sardinia (4%), with a mean allelic frequency across the whole Europe of 9.1% (Martinson, JJ, et al., 1997). But the *CCR5*Δ32 variant was found to be only 1% in individuals of African origin and 9.8% in Caucasians. (Lucotte G, et al., 1998).

[0008] *CCR5*Δ32 is a polymorphism of the *CCR5* gene that results in an abnormally truncated *CCR5* protein, which is not expressed on the cell surface. With but a few rare exceptions (Dean M, et al., 1996; Wu L, et al., 1997), individuals homozygous for the *CCR5*Δ32 allele are resistant to HIV-1 infection, while heterozygotes appear to exhibit a slower progression of disease (Biti R, et al., 1997; O'Brien TR, et al. 1997) and more favorable immunologic parameters (Cone, L.A, et al., 2000). Since recent reports imply an association of MS with HHV6 infection, a possible role of *CCR5*Δ32 mutation and HHV6 in MS was investigated. (Challoner PB, et al., 1995; Sanders VJ, et al., 1996; Knox KK, et al., 2000). An open reading frame within HHV-6 designated as U12 gene encodes RANTES (Isegawa Y, et al., 1998), which is a major ligand for HIV-1 co-receptor *CCR5* (Deng H, et al., 1996; Dragic T., et al. 1996) and blocks HIV-*CCR5* interactions (McDermott DH, et al., 2000; Stantchev, Broder, 2000). In MS, the presence of higher concentrations of RANTES in homozygotes compared to heterozygotes and both compared to wild type *CCR5*, could predispose someone to more aggressive CNS disease by HHV6 (Milne RS, et al., 2000). Further reports confirm that although HHV6 uses CD46 as a cellular receptor (Santoro F, et al., 1999), HHV6 binds to RANTES, which could

modulate a protective inflammatory response and assist in the spread of the virus by immune evasion (Knox KK, et al., 2000).

[0009] Previous studies have examined the role of the *CCR5* polymorphism in MS (Barcellos et al., 2000). Barcellos et al. showed that the presence of a *CCR5*Δ32 mutation resulted in a 3.2 year delay in the onset of familial MS, while Sellebjerg et al. (Sellebjerg F, et al., 2000) indicated that the age of onset of disease was somewhat lower (about 3 years) in patients carrying *CCR5*Δ32. Bennetts' et al. (Bennetts BH, et al., 1997) failed to demonstrate any difference.

[0010] Based upon the key role played by chemokines in the migration of macrophages and T cells in MS, the importance of genetic factors associated with, and the possible viral infection involved in, the pathogenesis of the disease, the role of the naturally occurring 32bp deletion (delta 32 allele) in the *CCR5* gene in patients with MS was investigated.

Summary of the Invention

[0011] In the present invention, an association was found to exist between survival rates of MS patients and the presence of a mutation in the CCR5 gene.

[0012] In one embodiment, the invention thus provides a method for predicting the survival rate or time of a subject having multiple sclerosis. The method comprises obtaining a sample from a subject having multiple sclerosis. The DNA of the sample is then evaluated for the presence of a mutation in the CCR5 gene, which mutation correlates to reduced survival of subjects having multiple sclerosis.

[0013] The mutation is preferably a deletion, and more preferably CCR5 delta 32.

Brief Description of the Figures

[0014] Figure 1 is a graph showing the frequency of the CCR5Δ32 mutant allele in MS patients in relation to years of survival (from age of onset to death).

[0015] Figure 2 is a graph showing Kaplan Meier product-limit survival curves for MS patients, separated by CCR5 11 vs. 12 or 22 genotypes.

Detailed Description of the Invention

[0016] The presence in MS patients of the CCR5 delta 32 mutation, while not predisposing the patient to developing MS, was concluded to influence the course of the disease.

[0017] 132 brain samples of deceased MS patients were examined for the CCR5 delta 32 mutation. Results showed that the presence of CCR5 delta 32 made the patients more susceptible to an earlier death. Specifically, observation of the 132 brain samples at autopsy showed that CCR5Δ32 did not protect patients from developing MS when compared to controls, but contrary to expectations, made the MS patients more vulnerable to accelerated disease and earlier death.

[0018] DNA was isolated from post mortem brain tissue samples of 132 non-Hispanic Caucasians with MS and from whole blood of 163 healthy subjects. Both groups were screened for the normal 1 allele and for the CCR5Δ32 deletion 2 allele, using the polymerase chain reaction (PCR). Alleles (1 and 2) and genotypes (11, 12 and 22) were counted and their distributions between groups

determined. Survival analyses were used to test the effect of CCR5Δ32 deletion on the survivorship.

[0019] A significant association of the CCR5 mutant allele was found with early death and with a progressive reduction in the years of survival from age of onset to the age at death with increased frequency of the CCR5 deletion allele (PL ≤ .00005). The death hazard ratio of CCR5 12 + 22 versus 11 genotype is 2.12, after adjusting for the clinical subtype, suggesting MS patients with CCR5 genotype 12 or 22 have twice the mortality as compared to the 11 genotype of patients. Interestingly, this effect is more significant in female MS patients than males, with the hazard ratio of 3.58.

[0020] MS subjects and controls were examined for differences in allelic frequencies and genotype prevalence. No significant variation was found among these groups. There was also no significant difference in age at onset of MS (data not shown). However, a marked difference was observed when the Chi-square test was used to examine the potential progressive increase in certain genotypes of MS subjects across five groups with years of survival: ≤ 5 years, 6-10 years, 11-15 years, 15-20 years and ≥21 years (see Table 1). The MS subjects with 11 genotype survived progressively more years compared to heterozygous subjects who survived progressively fewer years (P< .00001). Additionally, these groups were examined for possible differences in mutant allelic frequencies by years of survival. A significant association of the CCR5 mutant allele(2) with an early death is shown in Fig.1 with a progressive reduction in the years of survival from age at onset to

the age of death with increased frequency of the CCR5 deletion allele (PL= .00005).

[0021] The overall median survival time is 23 years (range 1-56) after the onset of MS. Comparing genotype 11 vs. 12/22 carriers, the median survival times are 24 and 16 years, respectively, while death rates are 0.039 and 0.056 for MS patients. Figure 2 shows Kaplan-Meier survival curves for patients with CCR5 genotypes. The cumulative death probability is significantly higher in the genotype 12 + 22 group than in the 11 group ($p \leq 0.00099$). For example, the cumulative death probabilities are 34% and 69% for the genotype group 11 and 12 + 22 after 20 years from onset. The 11 genotype was marginally significantly associated with the more benign clinical subtype ($p=0.033$, data not shown). Because the survival difference can be confounded by clinical MS subtype and the age of onset, the Cox regression model was used to test the main effect of CCR5 with simultaneous adjustment for these potential effects. The results showed that MS subtype was highly significant ($p \leq .0001$), while gender and age of onset were not. The hazard ratio was 2.12 for genotype 12 + 22 versus 11 with p value of ≤ 0.045 (Table 2A) after adjusting for MS clinical subtype. This means that MS patients with CCR5 genotype 12 + 22 have over twice the mortality as compared to the 11 genotype, even after adjusting for the subtype. Interestingly, this genotype effect mainly is seen in female patients (Table 2B). The hazard ratio in females was 3.58 (p-value ≤ 0.001; 95% CI= 1.74-7.38). A potential bias may exist because those patients with unknown subtypes may have worse clinical diagnosis and shorter survival years. However, this effect more likely

pushes estimates downward (i.e. more conservative) if these patients were associated with CCR5 genotype 12.

[0022] The above studies showed a significant gradient in survival for MS patients when individuals having mutant CCR5 genotypes were compared to those with wild type. Thus, the presence of CCR5 mutation in patients with MS showed an exactly opposite effect compared to the protective effect seen in patients with HIV-1 infection (O'Brien TR, et al., 1997; deRoda Husman A-M, et al., 1997).

[0023] Finally, the above results allow further speculation that an alteration in the immune response to stimulus (viral perhaps) occurs, whereby the response generated up-regulates RANTES, and the immune response is directed away from the TH1 to TH2 cells. The effects of certain cytokines such as IL-10 and 13 will dominate over IL-2, IL-12 and TNF-alpha. This polarization of T cells also alters their receptor expression and may be precipitated by the *CCR5*Δ32 mutation.

Table1

Frequency of CCR5Δ32 Genotypes in MS Patients in Relation to Years of Survival (Age at Onset to Death)

Years survived	CCR5 Genotypes 11 N	(%)	12 N	(%)	22 N	(%)	Total N
≤ 5	2	40.0	2	40.0	1	20.0	5
6 – 10	8	61.5	5	38.5			13
11 – 15	14	70.0	6	30.0			20
16 - 20	15	75.0	5	25.0			20
≥ 21	67	90.5	7	9.5			74
Total	106	80.3	25	18.9	1	.8	132

Chi Square	Value	df	Significance
Pearson Chi-Square	37.260	8	<. 00001
Linear-by Linear Association	16.463	1	. 00005

Table 2A

Effect of CCR5Δ32 on Survivorship in MS patients by Cox Proportional Hazard Model, with Adjustment of MS subtype (n=83)

Factors	Hazard ratio (SE)	Z	P-value	Lower 95%	Upper 95%
Onset age	1.03 (0.02)	2.00	0.045	1.00	1.06
CCR5	2.12 (0.56)	2.74	0.006	1.24	3.62
MS subtype	5.35 (1.44)	6.25	0.0001	3.16	9.06

Table 2B

Effect of CCR5Δ32 on Survivorship in Female MS patients by Cox Proportional Hazard Model, with Adjustment of MS subtype (n=50)

Factors	Hazard ratio (SE)	Z	P-value	Lower 95%	Upper 95%
CCR5	3.58 (1.32)	3.45	0.001	1.74	7.38
MS subtype	19.36 (10.24)	5.60	<0.0001	6.86	54.60

[0024] The invention is further illustrated by the following examples, which are not intended to be limiting.

EXAMPLE 1

Study material:

[0025] DNA was isolated from post-mortem brain tissue from 132 MS cases from the Human Brain and Spinal Fluid Resource Center at VA Los Angeles Health Care Center, Los Angeles, CA. All cases were necropsy-confirmed on the basis of white matter lesions and demyelination. The sample was comprised of 47 male and 85 female non-Hispanic Caucasians. The age distribution of MS cases had a range of 30-86, with a mean of 57.2; the age at onset of MS ranged from 18-57, with a mean onset age of 34.6. MS subtyping was obtained by chart review for 83 of the subjects, while the remaining charts provided insufficient data for accurate subtyping. Additionally, because a majority of the progressive forms are preceded by several years of relapsing-remitting course, and because the chart reviews relied more upon recent diagnostic information, the MS subtyping is probably biased toward more progressive forms. The control sample consisted of 163 adult college students from a nearby

university (78 males and 85 females), age range 18-49, mean age 34.3 years, from whom blood samples were obtained for genetic studies.

EXAMPLE 2

Genetic Analysis:

[0026] To detect CCR5 delta 32 mutations, genomic DNA was extracted from brain samples and whole blood (Example 1) by standard procedures. A PCR based assay was used to determine the presence of the CCR5 delta 32 deletion. The following oligonucleotides were designed to yield a 232 bp product for the wild type:

Forward- GRL012A* 5'-TGTTTGCGTCTCTCCCAG-3' and

Reverse- GRL012B* 5'-CACAGCCCTGTGCCTCTT-3'

[0027] PCR was performed using an initial denaturation step at 94^0C for 4 minutes, followed by a second denaturation step at 94^0C for 45 minutes, an annealing step at 55^0C for 45 minutes, an elongation step at 72^0C for 45 minutes for 29 cycles, and a final elongation step at 72^0C for 6 minutes. PCR products were run on 12% polyacrylamide gels at constant 150 V for 2 hours and stained with ethidium bromide and viewed on UV. The PCR product obtained had 232 bp for the wild type and a 32 bp deletion leading to 200 bp for the mutant allele. The data obtained on CCR5 delta 32 genotypes were subjected to statistical analysis.

EXAMPLE 3

Statistical Analysis:

[0028] Alleles and genotypes were counted and their distributions between groups were determined. The Chi-square test was employed to statistically compare these

groups. All statistical data calculations were done with the SPSS statistical package for Macintosh (release 6.1.1) (SPSS, Inc.; Chicago, IL). Survival analysis was applied to compare the survival time after MS onset between the *CCR5* genotypes. To estimate the survivor function, Kaplan-Meier product-limit was used (Kaplan EL, et al., 1958). Log-rank test was used to examine the differences between two survivor curves of two *CCR5* genotypes (11 versus 12 + 22). The Cox proportional hazard model (Cox DR, et al., 1984) was also applied to test the multivariate effects including *CCR5* genotypic effect, gender and MS age of onset. These analyses were done in STATA 7.0 (Stata Corp., College Station, Texas).

[0029] The above studies thus show a correlation between the CCR5 delta 32 deletion and early death by MS.

[0030] In accordance with the present invention, the CCR5 delta 32 deletion may serve as a prognostic marker for MS.

[0031] The publications and other materials used herein to illuminate the background of the invention, and to provide additional details respecting the practice of the invention, are incorporated herein by reference as if each was individually incorporated herein by reference.

[0032] While the invention has been disclosed by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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